Food Microbiology 48 (2015) 182–190

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Effect of exposure to stress conditions on propidium monoazide (PMA)-qPCR based *Campylobacter* enumeration in broiler carcass rinses

A. Duarte [a, b, *], N. Botteldoorn [b], W. Coucke [c], S. Denayer [b], K. Dierick [b], M. Uyttendaele [a]

[a] Laboratory of Food Microbiology and Food Preservation, Dept Food Safety & Food Quality Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium
[b] Institute of Public Health, Section Food-borne Pathogens, Juliette Wytsman Street 14, 1050 Brussels, Belgium
[c] Institute of Public Health, Section Medical Laboratories Quality, Juliette Wytsman Street 14, 1050 Brussels, Belgium

ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form
28 December 2014
Accepted 29 December 2014
Available online 20 January 2015

Keywords:
Propidium monoazide
Campylobacter
qPCR
Alternative method
Injured membrane / stressed cells

ABSTRACT

Campylobacter quantification by qPCR is unable to distinguish viable *vs.* dead cells in contrast to the culture-based ISO 10272-2 reference method. Propidium monoazide (PMA) has been used to overcome this disadvantage. A *Campylobacter* PMA-qPCR enumeration method was evaluated for its consistency and compared to the culture-based enumeration for both artificially and natural contaminated broiler carcass rinses. The PMA effect was further evaluated on stressed cells. Five conditions, commonly encountered during the slaughter process and storage (acid, heat, cold, oxidation and freezing), were inflicted to the broiler carcass rinses artificially contaminated with *Campylobacter jejuni* or *Campylobacter coli*. A better correlation between the reference method and the qPCR enumeration was obtained when PMA was used. The two cultured-based methods used showed a significant CFU reduction for heat, cold and acid stresses although the PMA-qPCR enumeration showed that viable bacteria were underestimated. Freezing showed the highest reduction effect, while the reduction extend was also overestimated by the microbiological enumeration procedure. Exposure to a mild oxidative stress was the only stress condition applied at temperatures permitting adaptation of *Campylobacter* and did not lead to either reduction in CFU nor in the PMA-qPCR signal.

1. Introduction

Campylobacteriosis is the most common human zoonosis in Belgium and European Union (EU) with respectively 6607 and 214 268 confirmed human cases and a EU notification rate of 55.79 per 100 000 inhabitants in the year 2012 (EFSA, 2014). The handling, preparation and consumption of broiler meat play an important role in human *Campylobacter* infections (EFSA, 2011; Habib et al., 2008; Humphrey et al., 2007; Silva et al., 2011). The *Campylobacter* status of poultry meat is traditionally assessed based on qualitative testing. However, the number of bacteria ingested is believed to be of great importance in relation to human illness (Nauta et al., 2009; Uyttendaele et al., 2006). In the EFSA (2011) Scientific Opinion on *Campylobacter* in broiler meat production it was estimated that by reducing the numbers of *Campylobacter* on the broiler carcasses by 1 log 10-unit a considerable public health risk reduction of campylobacteriosis in the EU would be accomplished. Despite this, no food safety criterion on *Campylobacter* in raw poultry meat has been established in the European legislation (European Comission, 2005). Hence, several EU Member States including Belgium, United Kingdom and The Netherlands have been using a level of 1000 CFU/g of *Campylobacter* as a target for broiler slaughterhouses to reduce the numbers of highly contaminated broiler carcasses.

The reference method for *Campylobacter* enumeration, the ISO 10272-2, relies on microbial culture techniques, and to obtain a result it takes at least 48 h. The development of a rapid and reliable method to enumerate *Campylobacter* that complies with the industry needs of requiring a faster result is necessary. *Campylobacter* quantification by real-time PCR (qPCR) has already been described

* Corresponding author. ISP-WIV, Juliette Wytsman Street 14, 1050 Brussels, Belgium. Tel.: +32 2 642 50 86; fax: +32 2 642 52 40.
E-mail addresses: Alexandra.Duarte@wiv-isp.be, Duarte.Alexandra@gmail.com (A. Duarte).

http://dx.doi.org/10.1016/j.fm.2014.12.011
0740-0020/

and it provides more rapid results (Botteldoorn et al., 2008; Josefsen et al., 2010; Schnider et al., 2010; Toplak et al., 2012). However, when compared to the reference method, qPCR is unable to detect just the viable cells given that dead cells' DNA is also amplified.

Reagents such as ethidium bromide monoazide (EMA) and propidium monoazide (PMA) have been used to overcome this disadvantage (Bae andWuertz, 2012; Banihashemi et al., 2012; Josefsen et al., 2010; Pacholewicz et al., 2013; Rudi et al., 2005). Both reagents have been reported as being able to enter cells that present a damaged membrane and to permanently attach to DNA after a light exposure. Consequently, only the DNA originating from intact membranes cells and most probably viable cells is left available in conditions to be amplified by PCR. Two studies about PMA application to *Campylobacter* enumeration in broiler meat have been published until now. The first one from Josefsen et al. (2010) presented a rapid tool for producing reliable quantitative data on viable *Campylobacter* bacteria in broiler carcass rinse. The developed method did not detected DNA from dead *Campylobacter* bacteria but recognized the infectious potential of the viable but non-culturable (VBNC) state and was thereby able to assess the effect of control strategies and provide trustworthy data for risk assessment. In 2013, the PMA was again studied in broiler carcass meat by Pacholewicz et al. (2013) but now along the slaughtering processing line. Unlike Josefsen et al. (2010), Pacholewicz et al. (2013) besides stating that the *Campylobacter* counts determined with the PMA-qPCR and the culture method were not concordant, founded limitations in PMA such as the incapacity of fully reducing the dead cells signal as well as a possible impact in the dye effect when cells are in high concentrations.

Like other bacteria, *Campylobacter* cells in naturally contaminated foods are frequently sublethally injured by the different conditions to which the food was exposed. In these circumstances, bacteria can become sensitive to agents that they would normally support. The reference ISO method for the enumeration of *Campylobacter* in broiler carcass meat uses a selective medium that contains substances that can be harmful to these injured cells. Jasson et al. (2007) found no difference in the *Campylobacter* enumeration between the non-selective and the selective media if no stress was induced, but an underestimation on the selective media was observed after the application of a stress. Also Ritz et al. (2007) confirmed that only uninjured cells (or very partially injured cells) were detected by the plate count method and as a consequence the survival of *Campylobacter* could be underestimated depending on the used method. Countries as Sweden and Norway are suggesting or using freezing of broiler carcasses as a control measure to reduce human campylobacteriosis (Hofshagen andKruse, 2005; Lindqvist and Lindblad, 2008). Although with freezing *Campylobacter* may be killed, a fraction may survive or be injured (Jasson et al., 2007) and not be detected by the reference method. Inevitably, in these conditions these may pose a residual risk towards the consumer.

To better understand PMA performance on sublethally injured *Campylobacter* cells, an enumeration PMA-qPCR method was tested on stressed cells, after development, validation and comparison to the culture-based method for both artificially and naturally contaminated broiler carcass rinse suspensions. Five different stress conditions expected to be met during the slaughtering process and storage, were inflicted to *Campylobacter jejuni* (*C. jejuni*) or *Campylobacter coli* (*C. coli*) artificially contaminated broiler carcass rinses and the developed method with and without PMA was compared with the reference method using two different selective media.

2. Materials and methods

2.1. Strains and incubation conditions

C. jejuni ATCC 33560 and *C. coli* ATCC 33561 were recovered on Columbia agar (Oxoid, England) from −80 °C in Laked Horse Blood (Sigma, England) and isolated on selective solid medium, modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, Denmark). Incubation conditions were at all times performed at 41.5 ± 1 °C under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, and 80% N_2) achieved using the Anoxomat (Mark II System, The Netherlands).

For the artificial inoculation, a pre-inoculum was made by inoculating one single colony in 10 mL of Mueller–Hinton broth (BioRAD, France) and was kept in the fridge under microaerobic conditions for one month. For each experiment, overnight cultures were prepared by inoculating 10 μL of this pre-inoculum in 10 mL of Mueller–Hinton broth. Before use, the growth of strains was measured at OD_{600}, using a conversion factor of 5×10^8 CFU/mL for an OD_{600nm} of 1 in the SmartSpec Plus Spectrophotometer (BioRAD, France). A concentration between 1×10^8 and 3×10^8 CFU/mL was assured and then adjustment to 1×10^6 CFU/mL was made.

2.2. Culture-based enumeration

The culture-based enumeration, for the broiler carcass rinse, consisted in the inoculation of 100 μL of the 10-fold serial dilutions in buffered peptone water (BPW, LAB M, United Kingdom) on Campyfood agar (CFA, BioMérieux, France) and mCCDA. For pure culture, besides the two already used media, an additional enumeration on Columbia agar was made.

The mCCDA medium plates were dried for 30 min prior to use to avoid swarming of the colonies.

2.3. Enumeration by qPCR

For the *Campylobacter* qPCR enumerations a translation of the obtained Ct value to the number of $\log_{10}$ CFU/g on the basis of the standard curve and dilution factors was made. In order to make the standard curve, an overnight *C. jejuni* culture was 10-fold serially diluted in BPW and culture-based enumerated in triplicate. All dilutions 10^{-2} to 10^6 CFU/mL were subjected to NucleoSpin® Food DNA extraction and went through 6 individual measurements by qPCR on 6 different days. An average of the 6 measurements of all dilutions was taken and a correlation between the Ct value obtained for different concentrations and the achieved culture-based enumeration in CFU/mL was established. The qPCR limit of quantification (LOQ) was expressed as the lowest concentration in the used range that gave a positive result with a probability of 0.95. The lowest tested concentration giving 6 positive on 6 measurements was considered as the LOQ of the qPCR. The full *Campylobacter* enumeration method LOQ was determined by taking in account all dilutions: 10 g of broiler neck skin in 40 mL of BPW, 2 mL of sample extracted in 100 μL of elution buffer and the qPCR enumeration on 1 μL of the eluted DNA.

The full *Campylobacter* enumeration theoretical limit of detection (LODt) which is the smallest amount of the target that can theoretically be detected was also calculated taking in account the already described dilutions.

2.4. Broiler carcass rinse preparation

The broiler carcass rinse was prepared according to the ISO 6887-2 recommendations. Ten grams of broiler carcass neck skin